Taming Amphotericin B

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Supporting information

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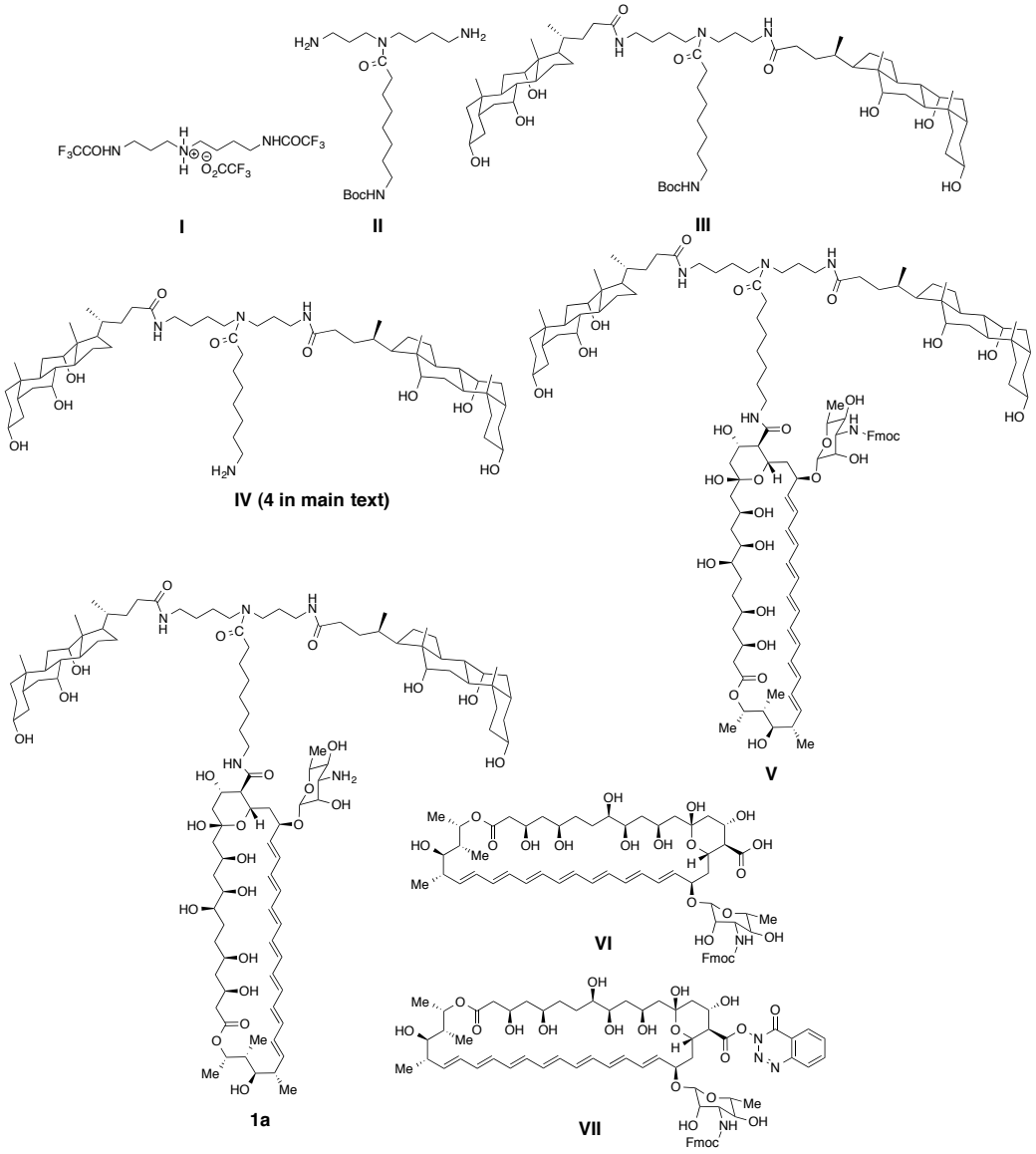
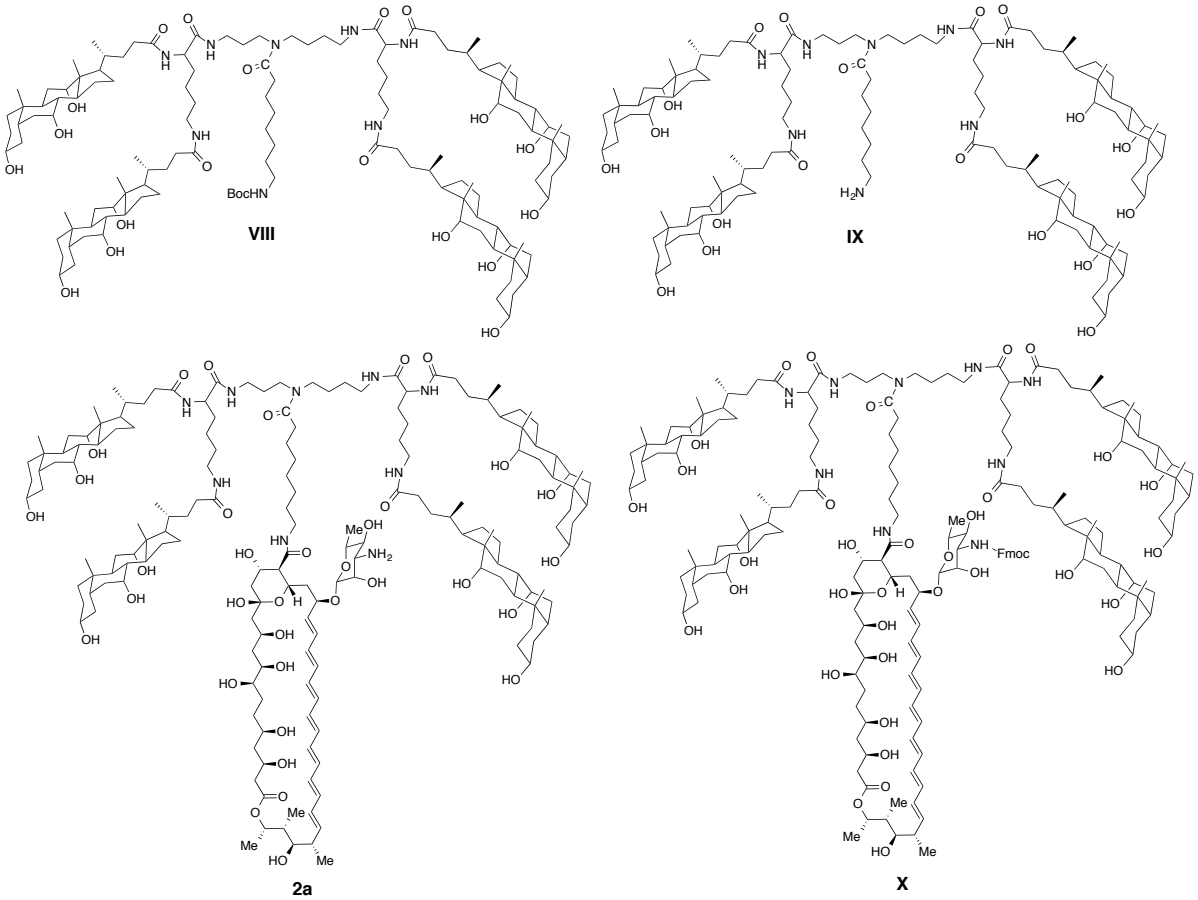
1. General information

Medium pressure liquid chromatography was performed using an Isolera One system equipped with a dual wave-length UV detector from Biotage and Biotage SNAP Ultra columns. For purification via preparative TLC, silica gel plates (EMD, 1 mm, 20 × 20 cm) were used, which contained a fluorescence indicator F254. Column chromatography was carried out using silica gel 60, EMD Milipore. All solvents were purchased from EMD Millipore Corporation and used as obtained. Deionized water was purified by a Millipore Milli-Q filtering system equipped with one carbon and two ion-exchange stages. All mass spectral measurements were performed by an Agilent LC-TOF high resolution TOF analyzer at the University of California-Riverside. All NMR spectra were recorded on a Bruker Avance 500 MHz instrument. Residual solvent signals were used as a reference. All UV measurements were performed on a Cary 300 Bio spectrometer from Varian.

2. Experimental procedures

2.1 Synthetic procedures





***N*$_2$-(Boc-8-aminocapryloyl)-spermidine amide (II).** N_2-(Boc-8-aminocapryloyl)-N_1,N_3-(trifluoroacetyl)spermidine triamide was synthesized in the following way: To a solution that was made from 271 mg (0.600 mmol) of spermidine N_1,N_3-bistrifluoroacetamide, TFA salt (**I**) (ref 1), 1.0 mL of DMF and 304 μL of *N*,*N*-diisopropylethylamine (1.90 mmol) (Sigma-Aldrich) was added a solution made from 0.155 g (0.600 mmol) of 8-Boc aminocaprylic acid (Chem-Impex Intl.), 1.5 mL of DMF, 229 mg (0.604 mmol) of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl uronium hexafluorophosphate (HBTU, Sigma-Aldrich), and 0.106 mL (0.612 mmol) of *N*,*N*-diisopropylethylamine. After the resulting mixture was stirred in closed flask at rt for 17 h, the volatiles were removed under reduced pressure (10 Torr, 45^{o}C). To the crude product mixture was added 30 mL of dichloromethane. The resulting solution was then washed, sequentially, with 5 mL of 0.5 M aqueous HCl, 5 mL of 2% $NaHCO_3$, and three times with 5 mL of H_2O, and then dried over anhydrous Na_2SO_4. Additional purification was carried out using a medium pressure gradient column chromatography (Isolera, Snap Ultra, 10 g, dichloromethane/isopropyl alcohol, 100/8 to 5/1, v/v) affording 0.265 g (76%) of N_2 N_2-(Boc-8-aminocapryloyl)-N_1,N_3-(trifluoroacetyl)spermidine triamide having R_f 0.65

(dichloromethane/ isopropyl alcohol, 5/1, v/v) and ^{1}H-NMR (MeOD): 3.28-3.40 (m, 8H); 2.99 (t, 2H); 2.33 (m,2H); 1.77 (m, 2H); 1.54 (m, 8H); 1.40 (s, 9H); 1.32(m, 6H).

Hydrolysis of N_2-(Boc-8-aminocapryloyl)-N_1,N_3-(trifluoroacetyl)spermidine triamide:
A solution was prepared from methanol (4.0 mL), 0.250 g (0.431 mmol) of this bis-trifluoroaceamide and 0.890 mL of 1N NaOH. After stirring this solution for 16 h at rt, the solvents were removed under reduced pressure (10 Torr, 35°C). The crude product was then dissolved in 15 mL of dichloromethane and washed three times with 2 mL of H_2O. The dichloromethane was then removed under reduced pressure and the product dried (0.5 Torr, rt, 24 h) to give 0.155 g (95%) of the spermidine derivative **II**, having ^{1}H-NMR (CD_3OD, ppm): 3.29-3.43(m,4H); 3.00 (t, 2H); 2.57-2.67 (m, 4H); 2.37 (t, 2H); 1.53-1.75 (m, 6H); 1.49-1.52 (m, 4H); 1.42 (s,9H); 1.32 (m,6H).

***N*$_1$,*N*$_3$-dicholeamido-N$_2$-(Boc-8-aminocapryloyl) spermidine triamide (III).** To a solution that was prepared from 179 mg (0.438 mmol) of cholic acid, 2 mL of DMF and 79 μL (0.455 mmol) of *N*,*N*-diisopropylethylamine was added 159 mg (0.455 mmol) of *N*,*N*,*N*',*N*'-tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uronium tetrafluoroborate (TDBTU, Fluka). After stirring the mixture at rt for 15 min in a closed flask, the solution of the activated ester of cholic acid was added to a solution that was made from 86.9 mg (0.224 mmol) N_2-(Boc-8-aminocapryloyl) spermidine amide (**II**), 0.80 mL of anhydrous DMF and 0.080 mL (0.461 mmol) of *N*,*N*-diisopropylethylamine. After stirring the mixture at rt for 16 h, it was added, dropwise, into 20 mL 2% aqueous $NaHCO_3$. The precipitate that formed was separated *via* centrifugation, and washed three times with 20 mL of H_2O. Subsequent freeze drying afforded 236 mg of crude product that was purified by gradient medium pressure chromatography (Isolera, SNAP Ultra 25 g, [CH_2Cl_2/MeOH/H_2O, 150/10/1 (v/v/v) to 85/15/1] to give 0.198 g (76%) of **III** having ^{1}H-NMR (CD_3OD, ppm): 3.94 (s, 2H); 3.79 (d, 2H); 3.34 (m, 6H); 3.20 (m, 4H); 3.00 (t, 2H); 0.91-2.40 (m, 78H); 1.42 (s, 9H); 0.70 (s, 6H).

***N*$_1$,*N*$_3$-dicholeamido-N$_2$-(8-aminocapryloyl) spermidine amide (IV and 4 in the main text).** A solution was prepared from 46.4 mg (39.6 μmol) of N_1,N_3-dicholeamido-N_2-(Boc-8-aminocapryloyl) spermidine amide (**III**), 2.5 mL of chloroform and 0.25 mL of trifluoroacetic acid and stirred in closed flask for 2 h at rt. The solvents were then removed under reduced pressure affording 46.3 mg of crude product. This product was then dissolved

in 2.0 mL of methanol. To this methanolic solution was then added 0.280 mL of 1N aqueous NaOH and the solution stirred at rt for 1 h. The solvents were then removed under reduced pressure, and the crude product dried (1 Torr, rt, 16 h). The resulting solid was triturated four times using 10 mL of H_2O and dried (1 Torr, rt, 4 h) to give 42.4 mg (99%) of the free amine (**IV**) having ^{1}H-NMR (CD_3OD, ppm): 3.92 (s, 2H); 3.76 (s, 2H); 3.20-3.43 (m, 6H); 3.17 (m, 4H); 2.92 (m, 2H); 0.83-2.38 (m, 78H); 0.69 (s, 6H).

Compound (V). A solution that was made from 2 mL of anhydrous DMF, 42.4 mg (39.7 μmol) of amine **IV** and 6.5 μL of triethylamine was added, dropwise, to a solution made from 52.7 mg (40.8 μmol) of N-Fmoc Amhotericin B carbamate, (3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (**VII**) in 0.500 mL of anhydrous DMF. The resulting mixture was stirred in closed flask for 5 h and then added dropwise into 45 mL of cold diethyl ether. The resulting solid was separated and triturated four times with diethyl ether. The crude product was purified by preparative thin layer chromatography (SiO_2, $CHCl_3$/MeOH/H_2O, 80/20/2, v/v/v) to give 46.8 mg (54%) of conjugate **V** having R_f 0.68 and ^{1}H-NMR (CD_3OD/$CDCl_3$, 10/1, ppm): 6.08-6.60 (m, 14H); 5.30-5.45 (m, 2H); 3.12-4.95 (m,28H); 3.92 (s, 2H); 3.76 (s, 2H); 0.89-2.58 (m, 111H); 0.69 (s, 6H).

N-Fmoc Amhotericin B carbamate, (3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (VII). (*N*,*N*,*N*',*N*'-Tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uronium tetrafluoroborate) (38 mg, 0.109 mmol) was added directly to a stirred solution that was made from Amphotericin B Fmoc carbamate **VI** (125 mg, 0.109 mmol) (ref. 2), 8 mL of DMF and 30 μL of *N*,*N*-diisopropyl ethylamine. The resulting mixture was stirred for 20 min at room temperature. After the reaction was completed (as judged by monitoring the formation of product by TLC, $CHCl_3$/MeOH/H_2O; 60/10/1, v/v/v, R_f 0.50), the mixture was added, dropwise, into 200 mL of diethyl ether and the yellow precipitate collected by centrifugation. The resulting solid was further purified by column chromatography using silica gel using $CHCl_3$/MeOH/H_2O (60/10/1, v/v/v,) as the eluent. The first major yellow band that was collected gave 121 mg (86%) of the title compound having ^{1}H NMR (500 MHz, CD_3OD/$CDCl_3$, 1/5, 25^{o}C, ppm): δ 8.37 (d, J = 7.5 Hz, 1H), 8.17 (d, J = 6.8 Hz, 1H), 7.90 (t, J = 7.7 Hz, 1H), 7.76 (m, 3H), 7.63 – 7.47 (m, 2H), 7.40 – 7.18 (m, 4H), 6.69 – 5.93 (m, 14H), 5.60 – 5.21 (m, 2H), 4.96 – 4.18 (m, 6H), 3.94 – 3.57 (m, 5H), 3.46 – 3.21 (m, 5H), 2.76 – 2.06 (m,5H), 2.04 – 1.25 (m, 17H), 1.22 (d, J = 6.3 Hz, 3H), 1.13 (d, J = 6.3 Hz,

3H), 1.03 (d, J = 7.1 Hz, 3H). HR-ESI MS for $C_{69}H_{86}N_4O_{19}[Na^+]$ Calcd: 1313.5728. Found: 1313.5752. **NOTE**: This compound should be stored in the dark at < 0°C.

Amphotericin B conjugate 1a. A solution was made from 21.2 mg (9.7 μmol) of compound **V**, 1.00 mL of DMF and 0.15 mL of piperidine and was stirred at 33°C for 2 h. After this time, the solution was added to 10 mL of cold diethyl ether. The solid that was formed was separated and triturated four times with 10 mL of diethyl ether and dried (1 Torr, rt, 16 h) to give 18.5 mg (97%) of **1a** having ^{1}H-NMR ($CDCl_3/CD_3OD$, 1/3, ppm): 6.08-6.60 (m,14H); 5.30-5.45 (m,2H); 3.12-4.95(m,25H); 3.92 (s, 2H); 3.76 (s, 2H); 0.89-2.58 (m, 111H); 0.69 (s, 6H). HR-ESI MS: for $C_{110}H_{182}N_5O_{25,}$ (MH^+) calculated: 1973,3033; found: 1973.3063.

***N*$_1$,*N*$_3$-bis(*N*$_1$'*N*$_2$'-dicholeamido-lysyl)-*N*$_2$-(Boc-8-aminocapryloyl) spermidine amide (VIII).** To a solution that was made from 0.251 g (0.27 mmol) of N_1,N_2-lysine dicholeamide (ref 3), 2.00 mL of anhydrous DMF, 51 μL (0.29 mmol) of N,N-diisopropylethylamine was added 0.1012 g (0.289 mmol) of TDBTU. After stirring the mixture for 15 min at rt in a closed flask, the solution of the activated ester was added to a solution made from 52.3 mg (0.135 mmol) of *N*$_2$-(Boc-8-aminocapryloyl)-spermidine amide (**II**) 0.60 mL of anhydrous DMF and 0.051 mL (0.29 mmol) of N,N-diisopropylethylamine. The resulting mixture was stirred for 24 h at rt and then added, dropwise, into 20 mL of 2% aqueous $NaHCO_3$. The precipitate that was formed was separated by centrifugation, washed three times with 20 mL of H_2O and freeze dried to give 300 mg of crude product that was purified by gradient medium pressure chromatography (Isolera, SNAP Ultra 25 g, CH_2Cl_2/MeOH/H_2O, 85 /15/1 to 80/20/2, v/v/v) to give 0.218 g (73%) of **VIII** having R_f 0.69 (CH_2Cl_2/MeOH/H_2O, 80/20/2, v/v/v) and ^{1}H-NMR (CD_3OD, ppm): 4.23 (m, 2H); 3.93 (s, 4H); 3.79 (s, 4H); 3.39 (m,12H); 3.18(m, 4H); 3.01 (m, 2H); 0.85-2.40(m, 150H); 0.70(s, 12H).

***N*$_1$,*N*$_3$-bis(*N*$_1$'*N*$_2$'-dicholeamido-lysyl)-N$_2$-(8-aminocapryloyl) spermidine amide (IX)**. A solution was made from 65.4 mg (29.7 μmol) of *N*$_1$,*N*$_3$-bis(-*N*$_1$'*N*$_2$'-dicholeamido-lysyl)-*N*$_2$-(Boc-8-aminocapryloyl) spermidine amide (**VIII**), 2.5 mL of chloroform and 0.35 mL of trifluoroacetic acid, and the resulting solution stirred in closed flask for 2 h at rt. After that time, the solvents were removed under reduced pressure and the crude product (80.3 mg) was

dissolved in 3.0 mL of methanol. To this solution was added 0.160 mL of 1 N aqueous NaOH and the solution stirred for 1.5 h at rt. The solvents were then removed under reduced pressure, the product mixture dried (1 Torr, rt, 16 h). The resulting solid was triturated four times with 2 mL of H_2O and dried (1 Torr, rt, 4 h) to give 58.5 mg (94%) of the free amine (**IX**) having R_f 0.75, ($CHCl_3$/MeOH/NH_4OH, 70/30/6, v/v/v) and ^{1}H-NMR (CD_3OD, ppm): 4.23(m,2H); 3.93(s,4H); 3.79(s, 2H); 3.39(m,12H); 3.18(m, 4H); 2.63(m, 2H); 0.85-2.40(m, 150H); 0.70 (s, 12H).

Conjugate X . A solution was made from 2 mL of anhydrous DMF, 58.3 mg (27.6 μmol) of amine **IX** and 4.5 μL (32 μmol) of triethylamine and the resulting solution then added dropwise to a solution of 37.2 mg (28.8 μmol) of N-Fmoc Amphotericin B carbamate, (3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (**VII**) in 0.500 mL of anhydrous DMF. After stirring the mixture in a closed flask for 6 h at 34^{o}C, it was then added dropwise into 25 mL of cold diethyl ether. The precipitate that was formed was separated and triturated four times with 20 mL of diethyl ether. The crude product (75 mg) was purified by column chromatography and by preparative thin layer chromatography (SiO_2, $CHCl_3$/MeOH/H_2O, 80/25/3, v/v/v) to give 29.1 mg (28%) of conjugate **X** having R_f 0.68 $CHCl_3$/MeOH/H_2O, 80/25/3 (v/v/v) and ^{1}H-NMR ($CDCl_3$/CD_3OD, 1/3, ppm): 6.08-6.60(m, 14H); 5.30-5.45(m, 2H); 3.12-4.95 (m, 35 H); 3.92 (s, 4H); 3.76 (s, 4H); 0.89-2.58 (m, 175H); 0.69 (s,12H).

Conjugate 2a. A solution was made from 29.1 mg (9.0 μmol) of conjugate **X** (9.0 μmol), 0.80 mL of DMF and 0.080 mL of piperidine, and stirred for 7 h at 33^{o}C. The solution was then added, dropwise, to 10 mL of cold diethyl ether. The solid that was formed was separated and triturated four times with 10 mL of diethyl ether and dried (1 Torr, rt, 16 h) to give 25.7 mg (95%) of **2a** having ^{1}H-NMR ($CDCl_3$/CD_3OD, 1/3, ppm): 6.08-6.60(m,14H); 5.30-5.45(m, 2H); 3.12-4.95 (m,32H); 3.92 (s, 4H); 3.76(s, 4H); 0.89-2.58(m, 175H); 0.69(s,12H). HR-ESI MS: for $C_{170}H_{281}N_9O_{35}$, (M+2Na) calculated: 1527.5135; found: 1527.5197

2.2 Critical Aggregation Concentration, Hemolytic Activity and Cytotoxicity Measurements.

Determination of Critical Aggregation Concentration (cac) of AmB and 1a.

Solutions of AmB and **1a** in DMSO (Sigma-Aldrich) were prepared having a concentration of 1.00 mM. Aliquots (1-10 μL) were then introduced into test tubes containing 0.750 mL to 5.88 mL of phosphate-buffered saline (PBS), pH 7.4 at 37°C to give concentrations ranging from 0.17 to 13.1 μM. After vortex mixing for 10 s, the solution was transferred to a 1.60 mL UV cuvette that was maintained at 37°C. The UV spectrum was then recorded in the range of 250-550 nm. The absorbance at 408 nm was plotted as a function of the reciprocal value of the concentration of AmB and the critical aggregation concentration of **1a** was then determined, graphically.

Hemolysis measurements. Sheep Red Blood Cells, 10%, in saline (Innovative Research, Novi, MI), were diluted saline (pH 7.4) to a concentration of 4%, which corresponds to 4 x 10^7 cells/mL. All measurements were carried out in duplicate. Solutions of Amphotericin B (Sigma-Aldrich) were prepared by adding 10 μL of a DMSO solution containing a given concentration of the antibiotic (i.e., ranging from 0.2 mM to 50 mM) to 490 μL of PBS. After the resulting solution was vortex mixed for 30 s it was incubated at 37°C for 15 min. The resulting solution of antibiotic (500 μL) was then mixed with 500 μL of the 4% erythrocyte dispersion at 37°C in a 1 mL plastic centrifuge vial to give a dispersion that was vortex mixed at slow rate for 5 s. After 1 h of incubation at 37°C, all samples were centrifuged (1500 g, Eppendorf centrifuge 5415C, 5000 rpm) at rt for 5 min. The supernatant (~ 0.7 mL) from each plastic vial was carefully separated from sediment using a disposable glass pipette and quickly transferred to test tube. A volume of 50 μL of this supernatant was then transferred to a UV cell containing 500 μL of PBS that was maintained at 37°C. The UV spectrum was then scanned (450-650 nm) and the absorbance at 575 nm used to determine the extent of hemolysis. The extent of 0% and 100% of hemolysis was obtained from A_{575} values of experiments that were run in absence of AmB (control, $[A_{575,}]_o$) and in presence, 100 μM AmB ($[A_{575}]_{max}$), respectively. The concentrations of AmB and **1a** that resulted in 50% release of hemoglobin, {[A575]max -[A575]o}/2, which are our EH_{50} values, were determined graphically.

Cell Culture of Human embryonic kidney HEK293:

Human embryonic kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL

streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C.

Inhibition of Cell Growth:

HEK293T cells were seeded in 96-well plates at a density of 6,000 cells/well and incubated overnight. Before treatment, compounds aliquots were solubilized in DMSO to obtain 10 mM stock solutions. Appropriate volumes of these stock solutions were added to DMEM media so the final concentration of DMSO is equal to 1%. After removal of cell media, 50 μL of treatment solutions were added to each well and incubated at 37 °C for 2 h. After treatment, the media was removed, and 100 μL of complete medium was added to each well before returning the plate to the incubator. Cell viability was determined after 72 h using the colorimetric MTT assay. Briefly, 10 μL of a 5 mg/mL MTT stock solution was added to the treated cells and incubated for 2 h at 37 °C. The resulting formazan crystals were solubilized in 200 μL of DMSO, and the absorbance was measured at 580 nm using an Infinite 200 PRO microplate reader (Tecan). Cell viability was calculated against control cells treated with the vehicle in DMEM.

Determination of MIC and MFC Values. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) are the lowest concentrations that are required for completely inhibiting growth, and killing at least 99% of the fungi, respectively. Specific procedures that were used were similar to those previously described (ref 4).

3. NMR Spectra of Compounds.

Figure SI-1: ^{1}H NMR spectrum of **VII** (**3** in main text).

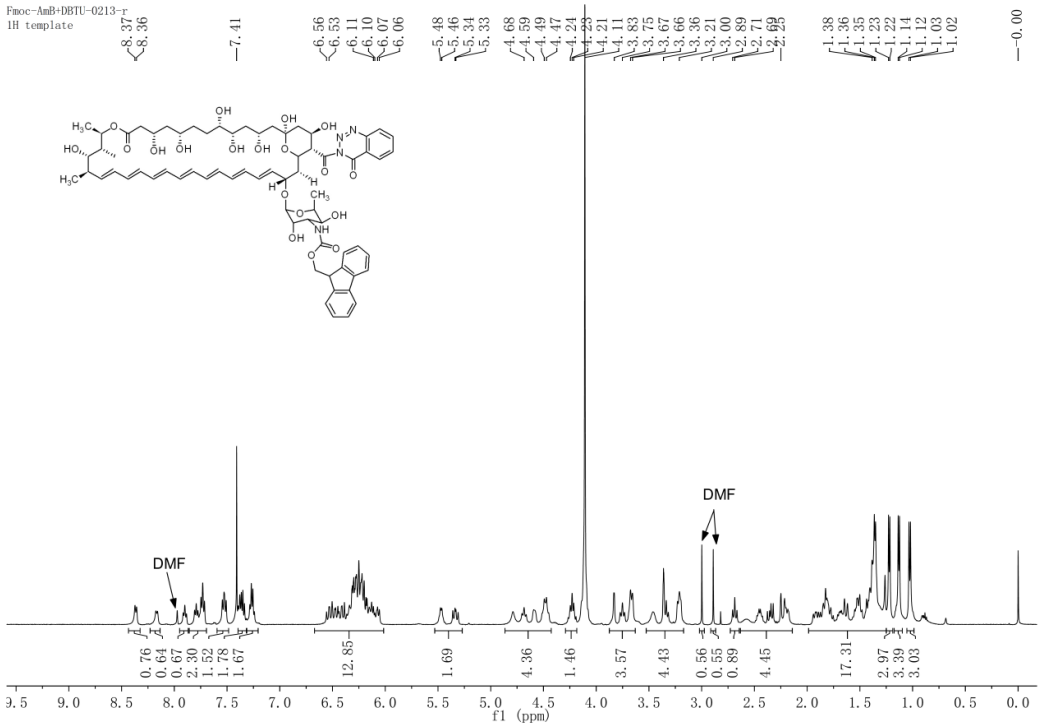


Figure SI-2: ^{1}H NMR spectrum of **1a**.

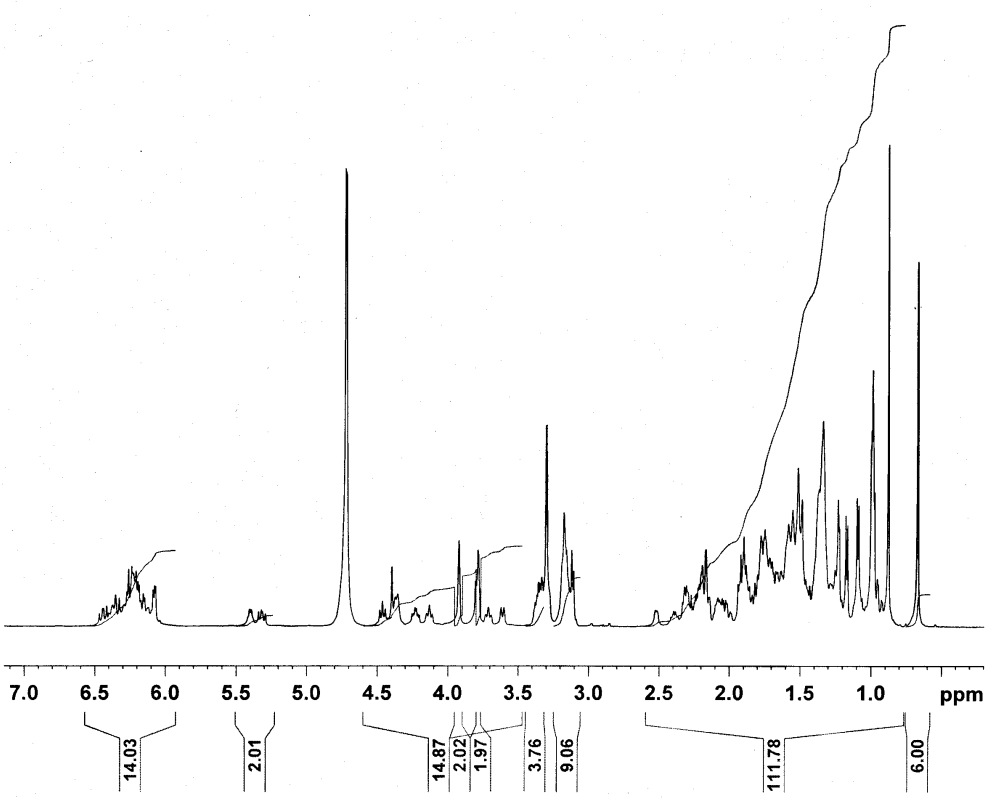
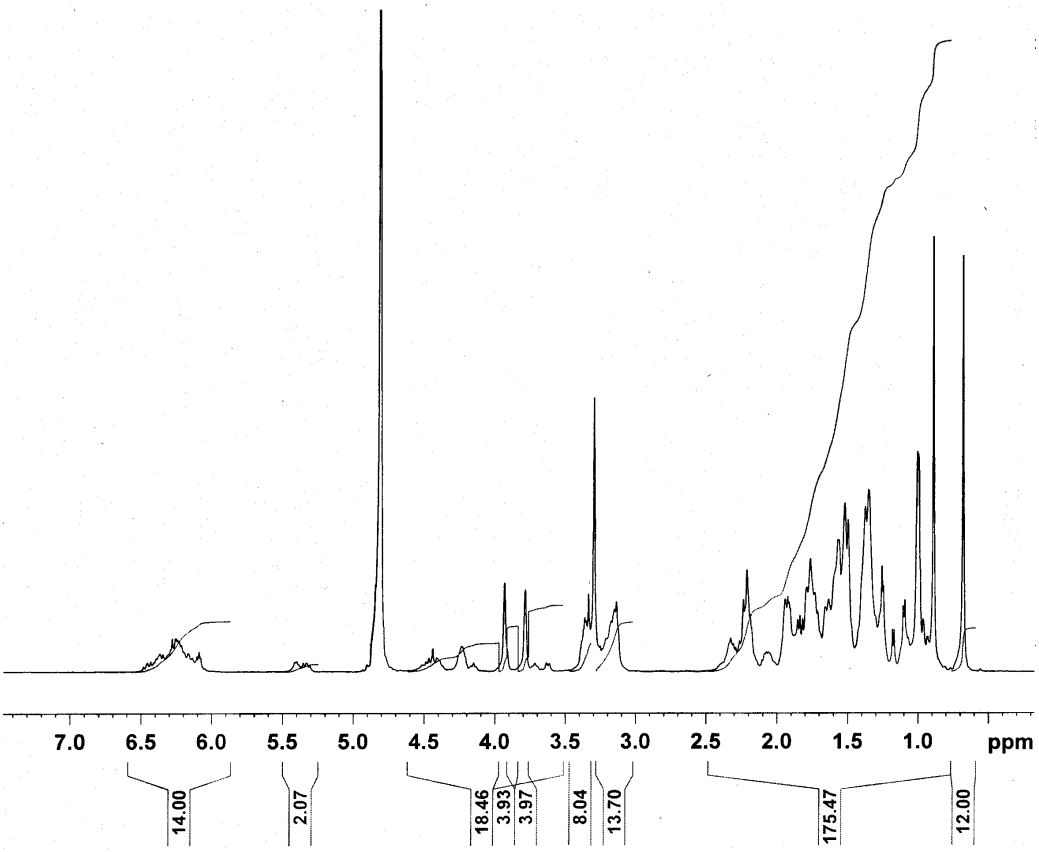


Figure SI-3: ^{1}H NMR spectrum of **2a**.



References.

1. O'Sullivan, M. C., and Dalrymple, D. M. (1995) A one-step procedure for the selective trifluoroacetylation of primary amino groups of polyamines. *Tetrahedron Lett. 36*, 3451-3452.

2. Palacios, D.S., Anderson, T.M., and Burke, M.D. (2007) A post-pks oxidation of the amphotericin b skeleton predicted to be critical for channel formation is not required for potent antifungal activity. *J. Am. Chem. Soc*. *129*, 13804-13805.

3. Chen, W. H., Shao, X.B. and Regen, S.L. (2005) Poly(choloyl)-based amphiphiles as pore-forming agents: transport-active monomers by design. *J. Am. Chem. Soc*. *127*,12727-12735.

4. Clinical and Laboratory Standards Institute (2008) Reference method for broth dilution antifungal susceptibility testing of yeasts, 3rd. Ed. CLSI document M27-A3 Clinical and Laboratory Standards Institute, Wayne, PA.